The Effects of Exogenous Polyamines and the Silencing of RLCK VI_A Kinases during Polar Growth of *Nicotiana tabacum* (Tobacco) Pollen Tubes

Summary of PhD thesis

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INTRODUCTION

Several genetic and/or environmental factors such as air, temperature, humidity can have harmful effect on fruit- and seed-set in seed plants. To minimize the harmful effect of these factors, it is important to get more information on the underlying genetics and physiology of pollen biology, including pollen viability and the regulation of pollen tube growth, which is required to increase the safety of crop productivity (Abdelgadir, Johnson, and Staden 2012). In this thesis, two different experimental approaches are described to better understand the role of polyamines and plant-specific Receptor like cytoplasmic kinases (RLCK VI_A), respectively, on pollen tube germination and growth in *Nicotiana tabacum*.

1. Polyamines (PAs): Polyamines (PAs) are naturally occurring in the cell as free and bound form and their polycationic nature is due to the spatial distribution of total charge. PAs play an important role in cell growth including pollen tube development in several species (Aloisi, et al. 2016; Pegg and Casero 2011). On the contrary, different studies have demonstrated the efficient action of PAs in reactive oxygen species (ROS) scavenging (Aloisi, et al. 2015). Exogenous application of PAs into pollen culture strongly affects pollen maturation, germination, and tube elongation depending on the dose and structure of PAs (Aloisi, et al. 2016; Aloisi, et al. 2015; Singh and Tandon 2012). Despite the number of studies about the role of PAs in pollen germination and growth, it is still largely unclear by which mechanisms PAs take part in the regulation of these processes. Although polyamines are known to affect reactive oxygen species (ROS) and Nitric oxide (NO) levels, we have limited information about the role of these compounds in polyamine-regulated pollen germination and pollen tube growth.

2. Receptor Like Cytosolic Kinases (RLCKs): The ROP (Rho of plant) GTPases are important regulators of plant cell polarity and as such play central role in the polar growth of pollen tubes. ROPs have many effector protein partners including potential ROP effector kinases, which belong to the large family of plant receptor-like cytoplasmic kinases (RLCK) namely to the class RLCK VI, group A (RLCK VI_A) (Dorjgotov et al. 2009; Jurca et al 2008). These kinases were shown to interact with ROPs and exhibit ROP-dependent in vitro

activity. There is, however, very limited information about the function of these potentially ROP-regulated receptor-like cytoplasmic kinases. Several members of the RLCK VI_A group exhibit strong expression in the pollen. Based on the above, these kinases might have function in the ROP-dependent growth of pollen tubes. In order to prove this hypothesis, the antisense oligodeoxynucleotide (AS-ODN) technology was used for the functional analysis of RLCK VI_A kinases during pollen tube growth in *Nicotiana tabacum*.

AIMS

One of the aims of the present work was to investigate if PAs, reactive oxygen species (ROS), and nitric oxide (NO) are interconnected in the process of pollen germination and tip-growth.

- To identify the effects of exogenous polyamines (Put; Putrescine, Spd; Spermidine and Spm; Spermine) on pollen germination and tube growth.
- To investigate how the inhibition of ROS production or ROS treatment effect the action of PAs on pollens/pollen tubes.
- To investigate how the application of NO scavengers or NO donors affect the germination/growth of polyamine-treated pollens.

Another aim of our work was to evaluate the function of the ROP-activated RLCK Class VI group A family protein kinase in pollen tube growth. It was supposed that these kinases should function in a similar way as ROP GTPases in the regulation of polar cell growth.

- To identify the members of the tobacco (*Nicotiana tabacum*) RLCK VI_A family
- To identify those RLCK VI_A genes of tobacco that are expressed in the pollen
- Designing and synthesis of antisense oligodeoxynucleotides (AS-ODNs) against the selected tobacco RLCK VI_A kinases.
- To investigate pollen tube growth and morphology in relation to the application of the synthesized AS-ODNs to pollen tubes

METHODS

- Growing *Nicotiana tabacum*, SR1) and *Arabidopsis thaliana* plants.
- Effect of exogenous polyamines on pollen growth and development.
- *In situ* detection of nitric oxide and reactive oxygen species with a fluorescent probe.
- Isolation of plant genomic DNA and total RNA.
- Investigation of gene expression levels with RT-PCR.
- Molecular cloning of RLCK VI_A genes cDNA.
- Sequencing of PCR amplified *Nicotiana tabacum* RLCK VI_A genes sequence.
- BLAST (Basic Local Alignment Search Tool) search of RLCK VI_A genes
- Construction of phylogenetic tree by the CLUSTAL OMEGA tool.
- AS-ODN designing and synthesis against the open regions of target mRNA.
- Delivery of AS-ODNs into growing pollen tubes.
- Measurements of pollen tube growth and tube length.
- Microscopic observation with Zeiss Axiovert 200 M-type fluorescent microscope and Bright field imaging with Olympus Cell-R microscope.
- Statistical analysis with ImageJ, SIGMAPLOT12.0 and GraphPad Prism version 6.

RESULTS

1. The various polyamines affect tobacco pollen grain germination dependent mainly on the level of ROS but not NO

The effects of the three polyamines Put, Spm, and Spd on the germination rate of tobacco pollens were different if applied at the same 10 µM concentration. While Put and Spd increased, Spm decreased pollen germination rate compared to the untreated controls.

To examine whether in the case of tobacco pollen, exogenous polyamines affected the germination rate through regulation of ROS and/or NO accumulation, the levels of these compounds were investigated in the treated pollen grains. None of the three polyamine treatments caused detectable changes in NO level. In contrast, Put and Spd decreased, but Spm increased ROS levels in pollen grains as compared to the controls. Since Put and Spd increased germination rate and Spm inhibited it, a correlation could be established between the action of the various PAs and the ROS accumulation. It was further strengthened by experiments where the co-application of Spm with the ROS scavenger DMTU (N, N'-Dimethylthiourea) reduced ROS production and increased the pollen germination rate as compared to the only Spm-treated pollens. Our data therefore suggest that polyamines differentially affect pollen germination according to their influence on the ROS, but not the NO level of tobacco pollen.

Exogenous polyamines affect tobacco pollen tube growth differentially altering the relative NO/ROS ratio: Considering the level of ROS and NO levels in growing pollen tubes, the NO level could be well correlated with the measured pollen tube length: Put decreased, but Spd increased NO production while Spm did not have a significant effect on it. We also observed that Put and Spm did not alter the ROS level of pollen tubes but the Spd treatment reduced it significantly in comparison to the untreated controls. To investigate that the observed changes in NO and/or ROS levels only correlates with or are the cause of the observed pollen tube growth phenotypes, the NO and ROS levels were manipulated in the polyamine treated pollen tubes. The NO-donor SNAP (S-Nitros-N-acetyl-D,L-

penicillamines) resulted in NO accumulation and complemented the negative effect of Put on pollen tube length without altering the ROS level.

In Spd-treated pollen tubes, the NO scavenger cPTIO (2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide) reduced the accumulation of NO and thus inhibited the positive effect of the treatment on pollen tube growth without affecting the ROS level. Interestingly, ROS accumulation due to exogenous H_2O_2 application reduced the endogenous NO level and concomitantly pollen tube length in Spd-treated pollen tubes in comparison to controls. These observations indicate that a high NO/low ROS ratio promotes while the opposite inhibits tobacco pollen tube growth and polyamines differentially affect this ratio primarily via decreasing (Put) or increasing (Spd) the NO level.

2. The role of RLCK VI_A family kinases in pollen tube growth and polarity

Nicotiana tabacum RLCK VI_A kinase cDNA sequences were screened for sequence homology (by BLAST) using the known cDNA sequences of Arabidopsis RLCK VI_A family members as queries. The gene expression analysis of the identified RLCK VI_A genes of *Nicotiana tabacum* showed variation in the level of gene expression. While the Nt RLCK VI_A1, Nt RLCK VI_A2, and Nt RLCK VI_A5, genes showed stronger expression in the leaf than in the pollen, all the others, namely Nt RLCK VI_A3, Nt RLCK VI_A4, Nt RLCK VI_A6, and Nt RLCK VI_A7 showed stronger expression in pollen than in the leaves.

The silencing of Nt RLCK VI_A members was achieved using specifically designed antisense oligonucleotides (AS-ODNs). It was observed that the silencing of Nt RLCK VI_A3, 4, 6 and 7 led to the formation of branches, knots, and bubble-like or widening tips of the pollen tubes. These abnormalities occurred in response to the AS-ODNs of all investigated Nt RLCK VI_As. In the case of control (without any treatment) and S-ODNs (sense strand; control), less than 2% abnormalities were registered, whereas in the case of AS-ODN treatments altogether 14 to 18% abnormalities were observed. Most of these abnormalities were branching. We found that antisense oligo treatment in parallel with the above polarity defects also caused reduction in the overall pollen tube length.

CONCLUSIONS

Pollen tube growth is a complex and well-coordinated process governed by various cellular and molecular pathways. In this thesis, the role of polyamines and the function of plant-specific kinases (RLCK VI_A) were evaluated during pollen tube germination and growth in *Nicotiana tabacum*.

1. The effect of different exogenous polyamines on tobacco pollen germination and growth was studied through their influence on ROS and NO production.

- Our results further support the involvement of PAs in the regulation of pollen germination and elongation affecting primarily ROS and/or NO levels
- The various polyamines inhibit pollen germination dependent on their effect on ROS production.
- In general, the relatively high NO/low ROS ratio promoted, while the opposite inhibited pollen tube elongation.
- Further investigations on the physiological function of PAs and their molecular partners are still needed. Especially as there is a wide variation in the reported action of PAs on pollen tube growth in various experimental systems and various plant species.

2. The role of ROP-activated Receptor Like Cytosolic Kinases (RLCKs) in pollen tube growth was investigated using antisense oligonucleotide-mediated gene silencing.

- Based on their pollen specific expression, four *Nicotiana tabacum* RLCK VI_A kinases were selected.
- Partial silencing of RLCK VI_A kinase members was attempted, using designed antisense oligonucleotides taken up into the growing pollen tubes.
- The observed phenotypes of the treated pollen tubes support the view that these kinases are indeed involved in the ROP GTPase-mediated polarity establishment and tip growth processes.

- However, further investigations need to be performed to verify this finding including the determination of the silencing efficiency and the specificity of the oligonucleotides in association with the observed phenotypes.

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PUBLICATION LIST

This PhD thesis is based on these articles

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+ These authors contributed equally to this work,

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OTHER PUBLICATIONS

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POSTER PRESENTATIONS

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EMBO/EMBL Symposium: Principles of Chromosome Structure and Function, EMBL, Heidelberg, Germany, 05-08 September 2018 (Poster). “Functional characterization of RLCKVI_A kinases with the usage of a miRNA-induced gene silencing system.”

TRAINING PROGRAM/ WINTER SCHOOL

Summer School (26-31 August 2018) at the BME Budapest University of Technology and Economics in the topic of Green cities.

FELLOWSHIPS

EMBO/EMBL fellowship for EMBO/EMBL Symposium: Principles of Chromosome Structure and Function, EMBL, Heidelberg, Germany, 05-08 September 2018.

Stipendium Hungaricum Scholarship for PhD program: Indo-Hungarian scholarship program.

OTHER FELLOWSHIPS

M.Sc. Teaching Fellowship: DBT, Ministry of Science and Technology India.

Summer Research Fellowship: Indian Academy of Science, Bangalore, Karnataka, India.

Biotech Industrial training fellowship: DBT, Ministry of Science and Technology India.

Senior Research Fellowship: Division of Genetics, Indian Agriculture Research Institute New Delhi, India.

CONFLICT OF INTEREST

The contributing authors of the above-mentioned peer reviewed publication and the unpublished part of this thesis declare that the authors have no conflict of interest related to this study. The supervisors also declare that the PhD candidate Shyam Jee worked under their supervision and he had a good contribution to the overall results evidenced in this thesis.

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Prof. Dr. Attila Fehér